Circulating Tumor Cells with Aberrant ALK Copy Number Predict Progression-Free Survival during Crizotinib Treatment in ALK-Rearranged Non-Small Cell Lung Cancer Patients

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Abstract

The duration and magnitude of clinical response are unpredictable in ALK-rearranged non–small cell lung cancer (NSCLC) patients treated with crizotinib, although all patients invariably develop resistance. Here, we evaluated whether circulating tumor cells (CTC) with aberrant ALK-FISH patterns [ALK-rearrangement, ALK-copy number gain (ALK-CNG)] monitored on crizotinib could predict progression-free survival (PFS) in a cohort of ALK-rearranged patients. Thirty-nine ALK-rearranged NSCLC patients treated with crizotinib as first ALK inhibitor were recruited prospectively. Blood samples were collected at baseline and at an early time-point (2 months) on crizotinib. Abarrent ALK-FISH patterns were examined in CTCs using immuno-fluorescence staining combined with filter-adapted FISH after filtration enrichment. CTCs were classified into distinct subsets according to the presence of ALK-rearrangement and/or ALK-CNG signals. No significant association between baseline numbers of ALK-rearranged or ALK-CNG CTCs and PFS was observed. However, we observed a significant association between the decrease in CTC number with ALK-CNG on crizotinib and a longer PFS (likelihood ratio test, P = 0.025). In multivariate analysis, the dynamic change of CTC with ALK-CNG was the strongest factor associated with PFS (HR, 4.485; 95% confidence interval, 1.543–13.030, P = 0.006). Although not dominant, ALK-CNG has been reported to be one of the mechanisms of acquired resistance to crizotinib in tumor biopsies. Our results suggest that the dynamic change in the numbers of CTCs with ALK-CNG may be a predictive biomarker for crizotinib efficacy in ALK-rearranged NSCLC patients. Serial molecular analysis of CTC shows promise for real-time patient monitoring and clinical outcome prediction in this population.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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second-/third-generation ALK inhibitors—ceritinib, alectinib, bri-gatinib, or lorlatinib—as well as combined treatment strategies with heat-shock protein 90 inhibitors (13, 18–23). Currently, there are no known predictive biomarkers allowing to identify those patients who will benefit from a long-term response to crizotinib from those whom are at risk of acquiring resistance shortly after starting crizotinib and would require alternate therapy promptly.

In current practice, predictive biomarkers for drug sensitivity and therapy selection are assessed either within the primitive tumor tissue taken at diagnosis or within tumor biopsies collected from metastasis during the course of the disease. Primitive tumor tissue can be difficult to obtain in patients with certain tumor types such as NSCLC where surgery is rarely a component of treatment. In NSCLC, genotyping and eligibility for targeted therapy are currently assessed using tumor biopsies, which can prove challenging to perform and obtain, are invasive, and may yield limited tissue quantities. Because tumors continually evolve and adapt to targeted therapies, it is becoming increasingly important in clinical practice to monitor the genetic changes occurring in tumors “in real-time” especially when selecting second- or third-line therapies. Subjecting patients to serial and repeated tumor biopsies for monitoring tumor evolution during treatment course and at the time of resistance is, in most cases, clinically not feasible. Therefore, finding alternative means to monitor tumor evolution and emergence of acquired resistance is a critical issue for improving the success rate of targeted therapies. Circulating tumor cells (CTC) can be obtained through a simple, non-invasive, and easily repeated blood draw (24–26). In contrast to tumor biopsies, CTCs have the potential to allow longitudinal monitoring of tumor features at different time-points during treatment, thereby guiding therapeutic decisions in a patient's clinical course. Another interesting characteristic is that CTCs are likely to be released from different metastatic sites and may have the potential to inform on the genetic heterogeneity of metastatic disease. Therefore, as such, CTCs may represent the metastatic disease as well as, if not better than a single-site tumor biopsy (25).

Using the CellSearch, a method based on the detection of EpCAM-positive epithelial cells, CTC levels were shown to have prognostic significance in several metastatic cancers, including NSCLC (25, 27). Longitudinal monitoring of changes in total CTC counts has also suggested a potential role for CTC in predicting whether a patient may benefit or not from anticancer treatment (27, 28). Few studies have, until now, demonstrated the feasibility of CTC assays for the detection of predictive biomarkers. Three groups, including our own, have reported ALK-rearrangement detection in CTCs using a size-based technique for CTC enrichment and FISH. The choice of this approach was justified by the higher number of CTCs recovered by this technique as compared with the CellSearch in NSCLC (29–31). Using ISET (isolation by size of epithelial tumor cells) and filter-adapted FISH (FA-FISH), we determined a cut-off value of four or more ALK-rearranged CTCs per 1 mL blood to predict whether ALK-rearrangement was present or not within the tumor tissue (29). In this study, several CTC subsets harboring distinct ALK-FISH patterns—including ALK-rearranged CTCs and CTCs with a gain of ALK-copy number (ALK-CNG)—were identified and were correlated with variable evolution on crizotinib treatment in the five examined patients (29). Based on this observation, we hypothesized that CTC subsets differing in ALK-FISH patterns might be associated with different clinical outcomes in ALK-rearranged patients treated by crizotinib. In the present study, we evaluated whether these CTC subsets monitored on crizotinib in an extended cohort of 39 ALK-rearranged patients could inform on treatment benefit. The association between ALK-rearranged and ALK-CNG CTC subsets and clinical parameters, including PFS and overall survival (OS), is presented herein.

Materials and Methods

Study design

The study (IDRCB2008-A00585-50) was conducted at Gustave Roussy (Villejuif, France), authorized by the French national regulation agency ANSM (Agence Nationale de Sécurité du Medicament et des produits de santé), and approved by the Ethics Committee and our Institutional Review Board. Stage IV ALK-rearranged NSCLC patients were recruited into the study between March 2011 and November 2014 and included 18 patients from our first study (29). Informed written consent was obtained from all patients. All patients received 250 mg crizotinib twice daily at initiation of therapy. Peripheral blood samples were collected at baseline and at an early time-point on crizotinib (usually 2 months depending on patient visits). All patients had a CT scan and/or MRI every 6 or 8 weeks. Consequently, CT scans and/or MRIs were performed at baseline and before the second time-point (median, 7 days before the second time-point; range, 1 to 49 days). ALK-rearrangement was tested in tumor by FISH, IHC, or reverse transcription (RT)-PCR (32).

Medical records were reviewed and clinical characteristics were collected retrospectively—including age, sex, smoking status, tumor histologic type, number of previous treatment lines before crizotinib, Eastern Cooperative Oncology Group (ECOG) performance status, number of metastatic sites, level of lymphocytes, PFS, and OS.

PFS was defined as the time between the date of the start of crizotinib and the date of clinical or radiological progression according to RECIST. OS was defined as the time between the date of the start of crizotinib and the date of death. Patients who were progression-free and alive at the time of analysis were censored.

CTC detection by CellSearch and enrichment by ISET

CTCs were enumerated using the CellSearch (Janssen Diagnostics, LLC) as previously reported (27, 33). CTC enrichment by ISET (Rarecells) was performed on 10 mL of blood as previously reported (29, 33).

Combination of immunofluorescent staining and ALK FA-FISH in ISET-enriched CTCs

Immunofluorescent staining (DAPI/CD45) and FA-FISH were performed on three ISET spots as previously reported (29, 34, 35). Scanning and analysis of immunofluorescent staining and FISH signals were performed on an ARIOL (Leica Biosystems Richmond Inc.) according to a previously reported sequential process briefly described in the Data Supplement (35). FA-FISH data came from the analysis of three independent ISET spots by combining immunofluorescent staining and ALK FA-FISH. Data presented result from the addition of the values of the three ISET spots. When a divergence ≥30% on one spot was observed compared with the two others, additional
spots were tested and data from spots with an inconsistent result were not considered.

Combination of immunofluorescent staining and FA-FISH using ALK break-apart probe combined with the chromosome 2-specific centromeric probe of ISET-enriched CTCs

Immunofluorescent staining of filters, FA-FISH, and scanning were performed as described below. The detection of the aneuploidy of chromosome 2 was performed using the Vysis ALK Break Apart Rearrangement Probe Kit combined with XCyting Centromere Enumeration Probe XCE 2 in blue (MetaSystems GmbH; 1:1). The FA-FISH method was established using ALK-rearranged NCI-H2228 cell line spiked into peripheral blood samples of healthy donors (Supplementary Fig. S1). The amplification was defined either by a cluster of isolated red signals (10) or ≥6 copies of ALK and a ratio of ALK to centromere copies superior to 2.5, whereas a ratio inferior to this with a copy number superior to 2 defines a CNG (36).

Scanning and image analysis of combined immunofluorescent staining and ALK FA-FISH in ISET-enriched CTCs

Scanning was performed on an ARIOL (Leica Biosystems Richmond Inc.) according to a sequential process, which includes: (i) scanning of filters after fluorescent staining, (ii) automated selection of DAPI+/CD45− cells, (iii) FISH scanning in DAPI+/CD45− cells, and (iv) analysis of FISH signals in DAPI+/CD45− cells. Specific FISH scanning settings have been established to detect a maximum number of FISH spots in filter-enriched CTCs, including 30 z-stacks, a distance of 0.6 μm between two z-stacks, and a multi-exposure protocol consisting of three separate exposure times for the green and red fluorochromes (35). Filters were analyzed by experienced operators (E. Pailler and M. Oulhen). The ALK-status was validated by a cytogeneticist (N. Auger). The dual-color FISH assay using the Vysis ALK Break Apart Rearrangement Probe Kit (Abbott Molecular Inc.) was performed as we previously reported (29). The ALK Break Apart Kit consists of two probes adjacent to the 3′ (red) and 5′ (green) ends of ALK. In cells with a native status of ALK, the overlapping of the probes results in a fused (3′, yellow) signal. The two characteristic ALK-rearrangement split patterns are the split of the 3′ (red) and 5′ (green) probes (a distance greater than two signal diameters is considered as a split), or an isolated 3′ (red) signal.

Cell lines

The NCI-H2228 cell line was obtained from American Type Culture Collection (ATCC CRL-5935, batch 3527839, obtained in March 2008). NCI-H2228 cells have been authenticated by us and repeatedly controlled for the presence of ALK-rearrangement by FISH testing. Recent FISH experiment for controlling the ALK-rearranged status of NCI-H2228 cells was performed on March 2016. NCI-H2228 cells were cultured in RPMI medium 1640 (Thermo Fisher Scientific Inc.) supplemented with 10% FBS (Thermo Fisher Scientific Inc.) and maintained in a humidified incubator in 5% CO2, at 37°C. NCI-H2228 cells were spiked at various dilutions into blood from healthy donors filtered on ISET, as done for patient samples.

Statistical analysis

Statistical analyses were performed using R software version 3.3.0 for Mac (packages MASS v-7.3.45 and survival v-2.39.3 were used; ref. 37) and SAS/STAT software version 9.4 of the SAS system for Windows (SAS Institute Inc.). ALK-rearranged and ALK-CNG CTC counts were considered as distinct scores, and descriptive statistics were calculated separately. Wilcoxon tests were used to compare CTC counts (i.e., ALK-rearranged, ALK-CNG) between groups, for each covariate. Linear regressions were performed to assess the correlation between CTC number at baseline and on crizotinib, for each gene alteration independently. Cox proportional hazard models were used to estimate HRs and 95% confidence interval (CI) for PFS and OS. Each continuous covariate was scored as 0 and 1, < median and ≥ median, respectively, except for age at baseline where a cut-off was applied: (0: <55 y/o; 1: ≥55 y/o). A stepwise backward/forward procedure was applied to the full multivariate model in order to objectively select the optimal predictors (38). For each statistical analysis, including Cox models, a P value ≤0.05 was considered significant.

Results

Detection of CTCs with aberrant ALK-FISH patterns at baseline and on crizotinib

Thirty-nine metastatic ALK-rearranged NSCLC patients treated by crizotinib as first ALK inhibitor were examined. Patient characteristics are presented in Table 1. Median age was 53 years (range, 25–81). A majority of patients were never or light smokers (67%) with adenocarcinoma tumor histology (97%). All patients had received one or more prior treatments. The mean duration of
crizotinib therapy was 15.4 months (median, 10.2 months; range, 0.8–48.1)–7 of 39 patients being censored as they were continuing treatment at the time of analysis. One patient (P6) received discontinuous crizotinib treatment because of hepatic toxicity. All 39 patients had a blood sample for ALK-rearrangement detection in CTCs at baseline. Among them, 29 patients had a blood sample drawn at an early time-point (usually 2 months, depending on patient visit) of crizotinib therapy. Ten patients were not monitored at this early time-point due to medical follow-up in another hospital. ALK-rearrangement was tested by FISH in tumor biopsies for all 39 ALK-rearranged patients. When useful to assess or confirm positivity, FISH was combined to IHC or RT-PCR (Supplementary Table S1). ALK-rearrangement was evaluated in ISET filtration-enriched CTCs from 3 mL blood using a recently developed two-step method that combined two-color (DAPI/CD45) immunofluorescent staining, FA-FISH, and semi-automated microscopy analysis (35).

Two main subsets of CTCs with aberrant ALK-FISH patterns were detected: ALK-rearranged CTCs and ALK-CNG CTCs (Supplementary Table S1, Fig. 1A). The ALK-rearranged CTC subset included CTCs with variable numbers of splits of the red and green signal (3’ and 5’) and of ALK-native copies (3’/5’≥2). In contrast to our previous study performed on a limited number of 18 ALK-rearranged patients (29), ALK-rearranged CTCs with an isolated red signal (3’) were rarely observed in 10 patients of this extended cohort (Fig. 1A). The number of ALK-native copies (3’/5’≥2) was highly variable in CTCs harboring only a CNG (Fig. 1A). Using a specific third-color FISH probe for the chromosome 2 centromere (Fig. 1B), the same number of centromere copies and centromeres were observed, confirming that these CTCs harbored a gain of chromosome 2 (i.e., a polysomy of chromosome 2) and not an amplification of the ALK-gene.

At baseline, the median numbers of ALK-rearranged CTCs and ALK-CNG CTCs were 14 per 3 mL (range, 1–119) and 12 per 3 mL (range, 1–53), respectively (Supplementary Tables S1 and S2). On crizotinib, median numbers of ALK-rearranged CTCs and ALK-CNG CTCs were 13 per 3 mL (range, 3–60) and 15 per 3 mL (range, 2–177), respectively (Supplementary Tables S1 and S2). Using CellSearch, median values of zero CTCs per 7.5 mL were detected both at baseline and on crizotinib (range, 0–713; range, 0–544), respectively (Supplementary Tables S1 and S2). Linear regressions showed a significant correlation between the number of ALK-rearranged CTCs at baseline and on crizotinib (R² = 0.278; P = 0.003), whereas no correlation was observed in ALK-CNG CTC numbers (R² = 0.017; P = 0.503; Fig. 1C). These results suggested that the number of ALK-CNG CTCs on crizotinib was independent from CTCs numbers at baseline and therefore possibly affected by treatment.

Association between CTC subsets and clinical parameters or outcome

Associations between ALK-rearranged or ALK-CNG CTC counts at baseline and clinical parameters are presented in Supplementary Table S3. A significant association was observed between the numbers of ALK-CNG CTCs and the smoking status (P = 0.002). All other clinical parameters were not significantly associated with ALK-rearranged or ALK-CNG CTC counts at baseline. After a median follow-up of 16.1 months (mean, 19.4 months), 33 of the 39 (84.6%) evaluable patients had disease progression according to RECIST criteria. Median PFS was 6.9 months (mean, 11.2 months; 95% CI, 2.4–9.7 months). Twenty-five (64.1%) patients died, resulting in a median OS of 16.7 months (mean, 19.9 months; 95% CI, 11.4–32.9 months).

For univariate analysis, counts of ALK-rearranged and ALK-CNG CTCs at baseline and on crizotinib were assigned into low/high groups according to their respective medians (Table 2, Supplementary Table S2). We did not observe a significant association between baseline ALK-rearranged CTCs or ALK-CNG CTCs and PFS (Table 2) or OS (data not shown). No clinical parameter was significantly associated with PFS (Table 2) or OS (data not shown).

Due to the noncorrelated counts of ALK-CNG CTCs at baseline and on crizotinib, we considered the dynamic change of these CTC counts under treatment as a possible parameter to evaluate. Patients were assigned into two groups: 16 patients presented stable or increased numbers of ALK-CNG CTCs (mean increase, 27; range, 0–169), whereas 13 patients presented a decrease of the same cells (mean decrease, −8; range, −18 to −2; Fig. 2A). PFS according to the dynamic change of ALK-CNG CTC numbers is presented in Fig. 2B. We observed a statistically significant association between the dynamic change of ALK-CNG CTC numbers on crizotinib and PFS on univariate analysis (likelihood ratio test, P = 0.025; Table 2). Median PFS was 14.0 months (95% CI, 2.3 months to NA) for patients presenting a decrease in ALK-CNG CTC numbers, and was 6.1 months (95% CI, 2.5–9.8 months) for patients with a stable or increased numbers of the same cells (Fig. 3). The dynamic change of ALK-CNG CTC numbers was not correlated with OS (Table 2, Fig. 3), which was most likely affected by subsequent treatment lines in this population after crizotinib therapy (Supplementary Table S4). No significant association between the dynamic change of ALK-rearranged CTCs on crizotinib and PFS (Table 2) or OS was observed on univariate analysis (data not shown).

Multivariate Cox proportional hazards regression analysis, using all the clinical covariates and CTC numbers, was significant for PFS (Table 2) but not for OS (data not shown; likelihood ratio test P values: 0.038 and 0.491, respectively). Smoking status, number of treatment lines, number of metastatic sites, and dynamic change of ALK-CNG CTC counts had significantly non-zero coefficients (P ≤ 0.05). This result was reinforced by a stepwise backward/forward optimization applied on the full model, which selected the same four covariates and improved the likelihood ratio test, P = 0.005 (Table 2; ref. 38). In this model, the dynamic change of ALK-CNG CTCs levels under crizotinib remained the most significant factor associated with the duration of PFS (HR, 4.485; 95% CI, 1.543–13.030, P = 0.006; Table 2). The Cox model for OS remained non-significant after the same optimization procedure (likelihood ratio test P value: 0.209).

Discussion

Herein, we examined aberrant ALK-FISH patterns in CTCs collected at initiation of crizotinib therapy from 39 ALK-rearranged patients. CTCs were classified into two subsets, including ALK-rearranged and ALK-CNG CTCs. Baseline CTC numbers in these two subsets were not predictive of crizotinib benefit. However, in 29 of these 39 patients monitored at an early time-point of crizotinib therapy, we observed a significant association between the dynamic evolution of the numbers of ALK-CNG CTCs on crizotinib and median PFS of patients. These results demonstrate for the first time that molecular analyses of CTCs can identify a
Figure 1.
Detection of CTCs harboring ALK gene aberrations at baseline and on crizotinib therapy in ALK-rearranged NSCLC patients. A, Examples of ALK-rearranged CTCs and CTCs with ALK-CNG detected by combined fluorescent staining and FA-FISH. Red and green arrows show the ALK 5' and the ALK 3' probes, respectively. Scale bars, 10 μm. B, Examples of ALK-rearranged and ALK-CNG CTCs detected by combined fluorescent staining and three-color FA-FISH for ALK gene and chromosome 2 centromeric detection. Scale bars, 10 μm. C, Linear regressions for ALK-rearranged and ALK-CNG CTCs at baseline and on crizotinib therapy.
Serial Analysis of CTCs in ALK-Rearranged NSCLC

Table 2. Univariate and multivariate cox regression analysis for prediction of PFS

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age at baseline (y/o), ≥55 vs. &lt;55</td>
<td>0.602 (0.292–1.244)</td>
<td>0.171</td>
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<tr>
<td>Sex, M vs. F</td>
<td>1.575 (0.686–2.755)</td>
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<tr>
<td>Smoking status (PY), ≥15 vs. &lt;15</td>
<td>1.450 (0.685–2.938)</td>
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<tr>
<td>Number of previous treatment, 2 vs. 1</td>
<td>0.924 (0.464–1.840)</td>
<td>0.822</td>
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<tr>
<td>ECOG PS at baseline, ≥1 vs. &lt;1</td>
<td>1.926 (0.784–4.732)</td>
<td>0.153</td>
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<tr>
<td>Number of metastatic sites, ≥2 vs. 1</td>
<td>1.535 (0.760–3.020)</td>
<td>0.233</td>
</tr>
<tr>
<td>Metastases at baseline, liver</td>
<td>brain</td>
<td>both vs. none</td>
</tr>
<tr>
<td>ALK-rearranged CTCs at baseline, ≥1 vs. &lt;1</td>
<td>0.832 (0.417–1.658)</td>
<td>0.600</td>
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<td>ALK-CNG CTCs at baseline, ≥13 vs. &lt;13</td>
<td>1.676 (0.729–3.853)</td>
<td>0.224</td>
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<td>ALK-CNG at baseline, ≥12 vs. &lt;12</td>
<td>1.071 (0.511–2.024)</td>
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<tr>
<td>ALK-CNG on crizotinib, ≥15 vs. &lt;15</td>
<td>1.817 (0.830–4.077)</td>
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<td>Dynamic change of ALK-rearranged CTCs, stable/increase vs. decrease</td>
<td>2.059 (0.838–5.058)</td>
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<tr>
<td>Dynamic change of ALK-CNG CTCs, stable/increase vs. decrease</td>
<td>2.768 (1.097–6.988)</td>
<td>0.031</td>
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</table>

Optimized Model

- Smoking status (PY), ≥15 vs. <15
- Number of previous treatment, 2 vs. 1
- Number of metastatic sites, ≥2 vs. 1
- Dynamic change of ALK-CNG CTCs, stable/increase vs. decrease

Abbreviations: LR, likelihood ratio; PS, performance status; PY, pack-year; y/o, years old.

Predictive biomarker of early progression on crizotinib treatment in ALK-rearranged NSCLC patients. Further studies are needed to confirm our results and evaluate whether these findings may be extended to other ALK inhibitors. Although our data should be interpreted in the context of a small cohort, our study offers a promising perspective on the potential use of CTCs, which are accessible by a simple blood test, to monitor tumor genetic characteristics and to serve as a predictive biomarker of treatment efficacy among this lung cancer population.

In our first study, ISET-filtration, FA-FISH, and manual microscope analyses were used to detect ALK-rearrangement in CTCs captured in 1 mL blood (one ISET spot) from 18 ALK-positive and 14 ALK-negative patients (29). All ALK-positive patients had four or more ALK-rearranged CTCs per 1 mL blood, whereas no or only one ALK-rearranged CTC was detected in ALK-negative patients (29). One ISET spot contains on average 5,000 white blood cells (mainly granulocytes), around 70 CD45-negative cells, and a small number of CTCs. Consequently, the manual analysis of one ISET spot to identify ALK-rearranged CTCs is laborious, time-consuming and may be operator-dependent. In order to analyze larger blood volumes (i.e., more ISET spots per blood sample), limit intrasample variations (each spot representing an independent FA-FISH test), and reduce interoperator differences, we made technical changes and automated the microscope and imaging analyses. Also, in order to reduce the number of cells to be analyzed and render the analysis accessible by a scanner, we combined immunofluorescent staining and FA-FISH, and developed a specific automated FISH scanning protocol so as to limit FISH scanning to CTC candidates (DAPI/CD45 cells; ref. 35). The latter procedure was optimized for filters using 30 z-stacks spaced 0.6 μm apart as well as a multixposure protocol for optimal capture and definition of FISH signals (35). With this new semiautomated FISH analysis method, we were able to analyze larger blood volumes (three independent ISET spots), limit intrasample variations, and subsequently improve the precision of the assay. Using this improved technical approach, approximately 80% of the patients included had at least four ALK-rearranged CTCs per 1 mL blood. This discrepancy from prior observations in our first study could be explained by a difference between spots of the same sample or a possible underestimation of some FISH signals (due to insufficient number of z-stacks in the case of very large CTCs) during the semiautomated analysis. Moreover, it must also be acknowledged that, in some cases, an automated protocol cannot completely match the manual and case-per-case microscope adjustment performed by experienced operators or cytogeneticians. However, in spite of this consideration, the method used here increased the feasibility, precision, and reliability of ALK-FISH testing in filtration-enriched CTCs and

Figure 2.
Correlation between the dynamic change of the numbers of CTCs with an ALK-CNG on crizotinib and survival in ALK-rearranged patients. A, Dynamic change of the numbers of ALK-CNG CTCs on crizotinib. B, PFS according to the dynamic change of the number of ALK-CNG CTCs.
may contribute to progress in the validation and qualification of CTC assays in ALK-rearranged NSCLC. Finally, it is worth noting that other noninvasive approaches, such as circulating free DNA assays, are currently available for detecting gene rearrangements and gain of copy numbers, although the sensitivity and precision of these strategies need to be further evaluated and compared with those of CTC assays.

In ALK-rearranged NSCLC treated by crizotinib as first-line treatment, the median PFS is 9.7 months, but the duration and magnitude of clinical benefit vary widely between patients ranging from a few months to several years (5). The biological mechanisms underlying the heterogeneity in crizotinib responses are unknown (17). Extensive analyses of diagnostic tumor biopsies according to ALK-FISH patterns performed prior to crizotinib therapy have failed to identify informative parameters associated with treatment benefit (39, 40). Our results are in agreement with these data in demonstrating that baseline numbers of ALK-rearranged and ALK-CNG CTCs were not predictive of crizotinib benefit. One of the earliest mechanistic hypotheses for the heterogeneous responses to crizotinib was the differential sensitivity of the different EML4-ALK variants (41–43). Recently, in a retrospective study of 35 patients, patients positive for ALK-variant 1 in tumor specimens were reported to have a significantly longer PFS to crizotinib than patients with nonvariant 1, a result that differed from in vitro studies showing that ALK-variant 2 was the most sensitive to ALK-inhibitors, variant 1 having an intermediate sensitivity (42, 44–46). Here, we report that the dynamic change on treatment of a CTC subset with an aberrant ALK-CNG can identify patients who will experience a durable response to crizotinib. In tumor biopsies from ALK-rearranged patients, ALK-CNG is frequent but in most cases limited to three or four ALK-copies. The presence of ALK-CNG tumor cells was also associated with aggressive clinical outcome in several cancers such as neuroblastoma, breast, and colorectal cancer, with most ALK-CNG co-occurring with other gene aberrations such as MYCN, MET, or EGFR (36, 47–50). Hyperploid tumor cells harboring a high number of ALK-copies are rarely found in tumor biopsies from ALK-rearranged patients but are a large contingent of CTCs. Herein, we have clarified the FISH status of these CTCs by three-color FISH experiments using a chromosome 2 centromere probe and have shown unambiguously that CTCs harbor a gain of the native ALK-gene and not an amplification. The rarity of these hyperploid cells in tumor biopsies from ALK-rearranged patients might explain why these have been unexplored.

The results of our study could appear unintuitive to the extent that one might expect an association between therapeutic outcome and ALK-rearranged CTCs rather than ALK-CNG CTCs. Cells bearing ALK-CNG have been identified in two cases of 11 resistance biopsy specimens, whereas another study reported high-level amplification of ALK-fusion in one case of 15 patients (9, 10). Consistent with earlier studies, it was recently reported that only a minority of ALK-rearranged patients (~20%) developed ALK resistance mutations on crizotinib therapy, implying that other mechanisms, such as gain or amplification of ALK-gene, upregulation of bypass signaling pathways (e.g., EGFR, KIT, MEK/ERK), or histologic transition (e.g., epithelial–mesenchymal transition, EMT), may be dominant resistance mechanisms (15, 17). Such pathways or biological processes may be activated in hyperploid tumor cells with aberrant ALK-CNG, which could be more prone to genomic instability and therefore to acquiring tumor-promoting alterations. Therefore, our results suggest that the increased migration under crizotinib therapy of CTCs with ALK-CNG—possibly harboring resistance alterations in other pathways than ALK and increased metastatic potential (due to this secondary activation)—is associated with poor benefit and a risk of progression shortly after starting treatment. Resistance to ALK-inhibitor is heterogeneous, and single-site biopsies may be unable to represent the full scope of resistance alterations. Issued from different metastatic sites, CTCs are enriched in cancer cells with specific biological characteristics, such as EMT properties or tumor-initiating cell capacity, and may be a valuable tool to evaluate tumor heterogeneity and monitor resistance alterations in real-time.

We report for the first time that serial molecular analysis of CTCs could identify a predictive biomarker of therapeutic efficacy in ALK-rearranged NSCLC and could help to stratify patients at risk of early resistance. Although further confirmatory studies are needed, our results offer a new and important perspective on the potential use of CTCs in replacing biopsies for patient monitoring in real time and clinical outcome prediction in this population.

**Disclosure of Potential Conflicts of Interest**

D. Plancheaud is a consultant/advisory board member for AstraZeneca, IMS, MSD, Novartis, Pfizer, and Roche. J.-C. Soria is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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**References**


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